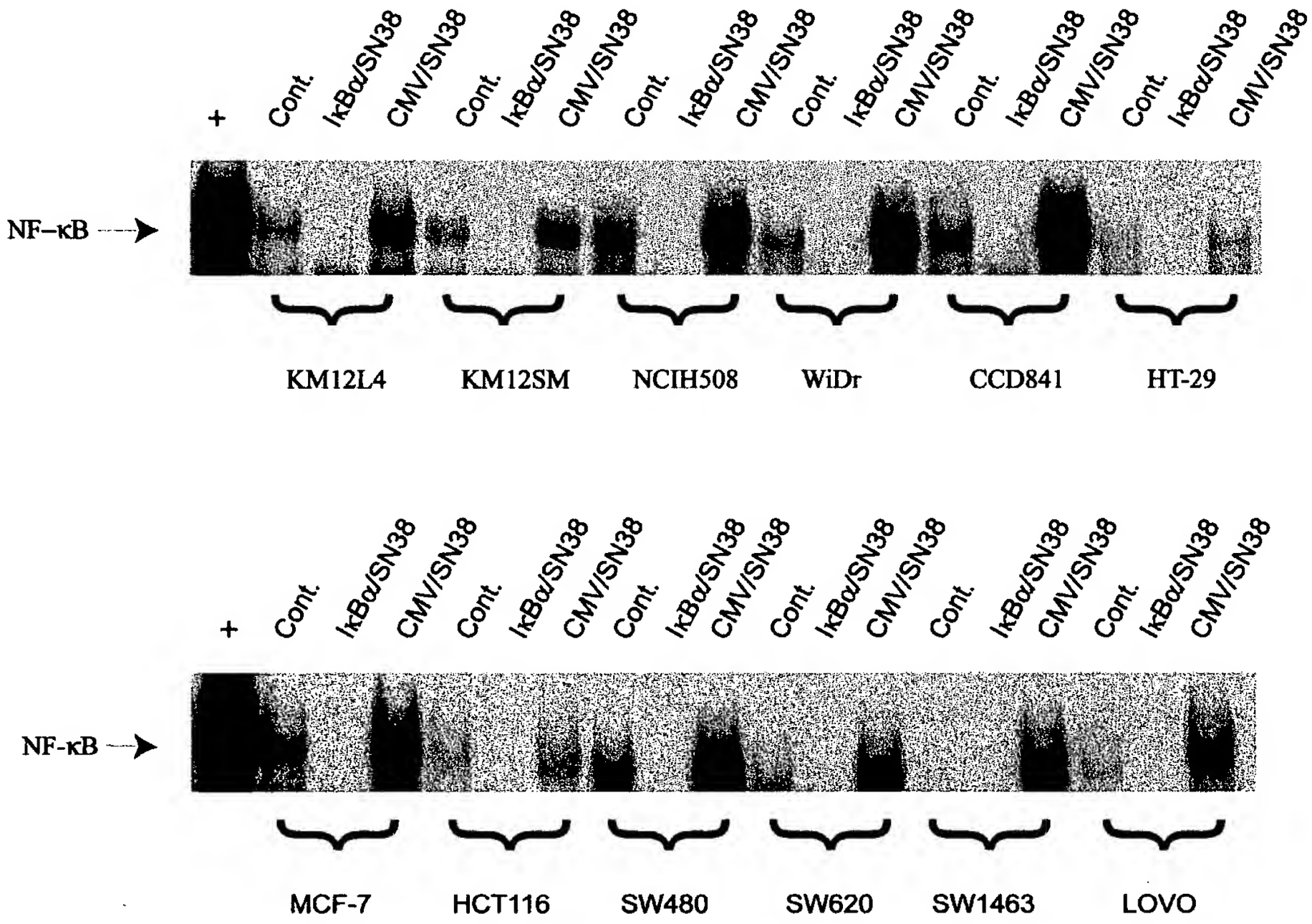
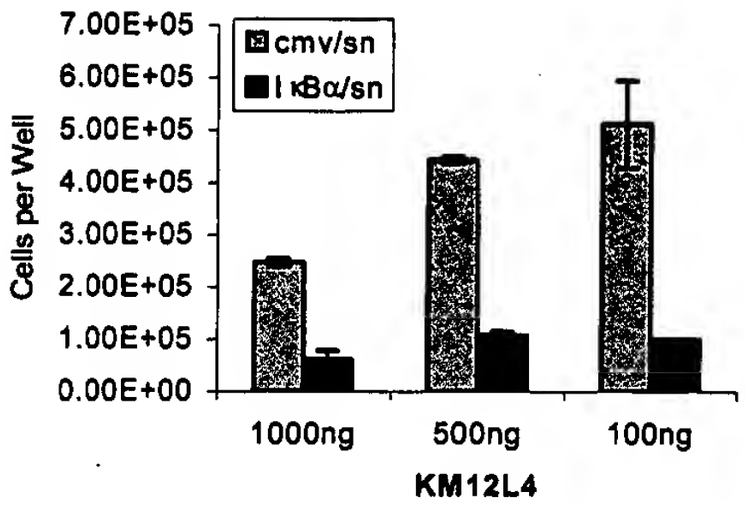

+
Cont.
IκBα/SN38
CMV/SN38
NF-κB
KM12L4
KM12SM
NCIH508
WiDr
CCD841
HT-29
MCF-7
HCT116
SW480
SW620
SW1463
LOVO

Cusack, JC

Fig 1A (copy)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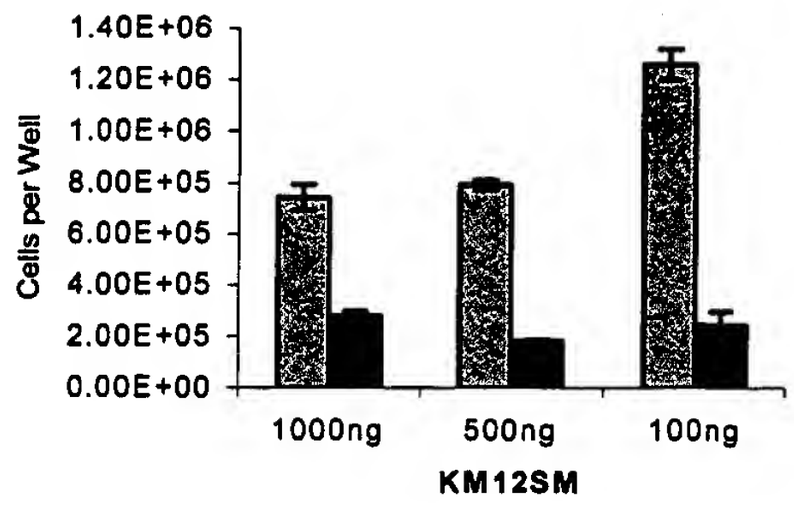
Cells per Well
cmv/sn
I κBα/sn
1000ng
500ng
100ng
KM12L4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Cells per Well
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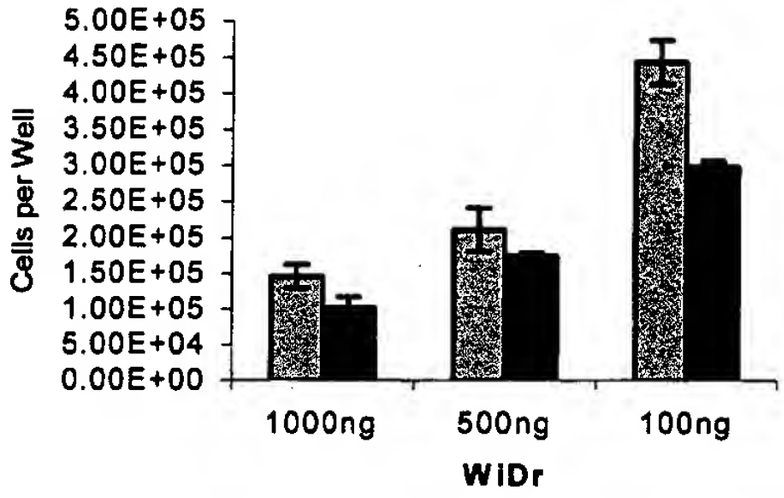
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Cells per Well
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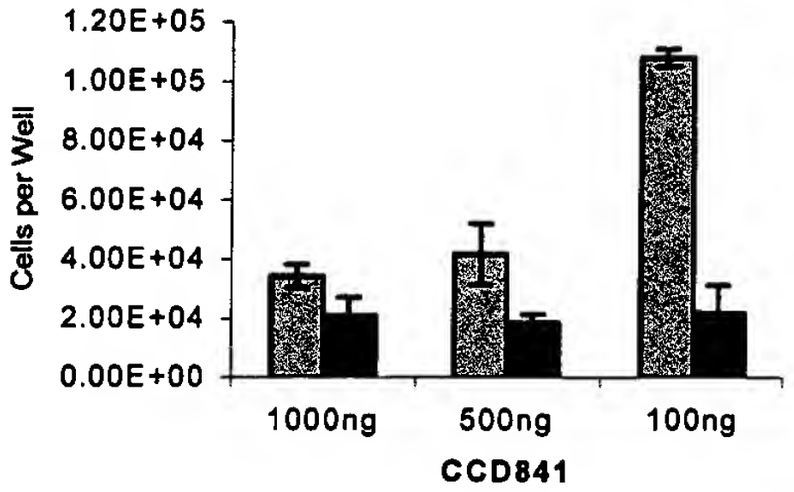
WiDr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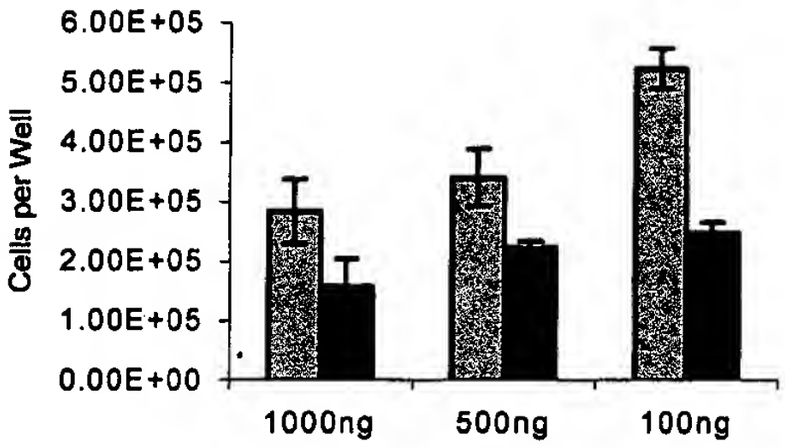
500ng
100ng
CCD841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Cells per Well
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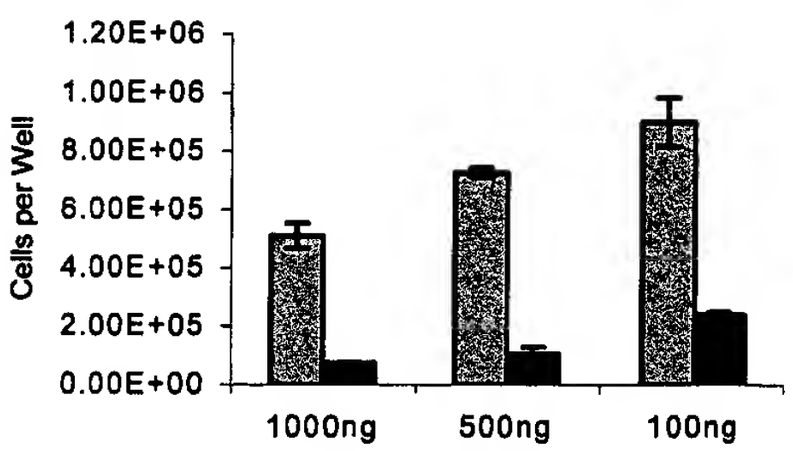
500ng
100ng
MCF-7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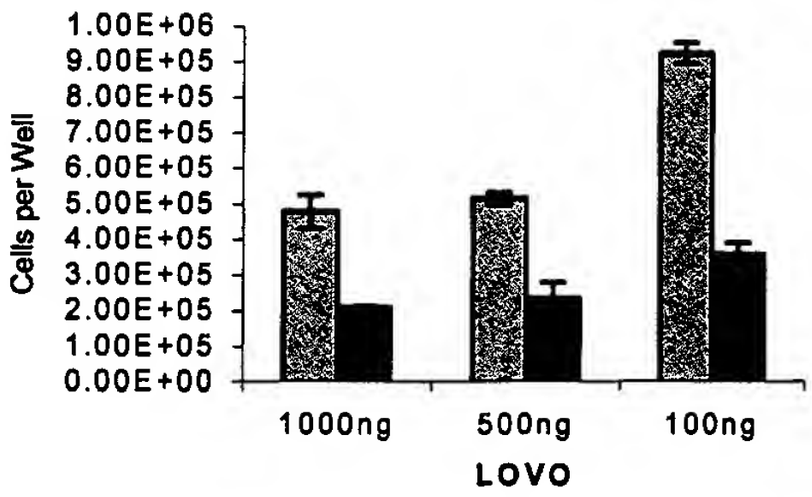
500ng
100ng
HCT116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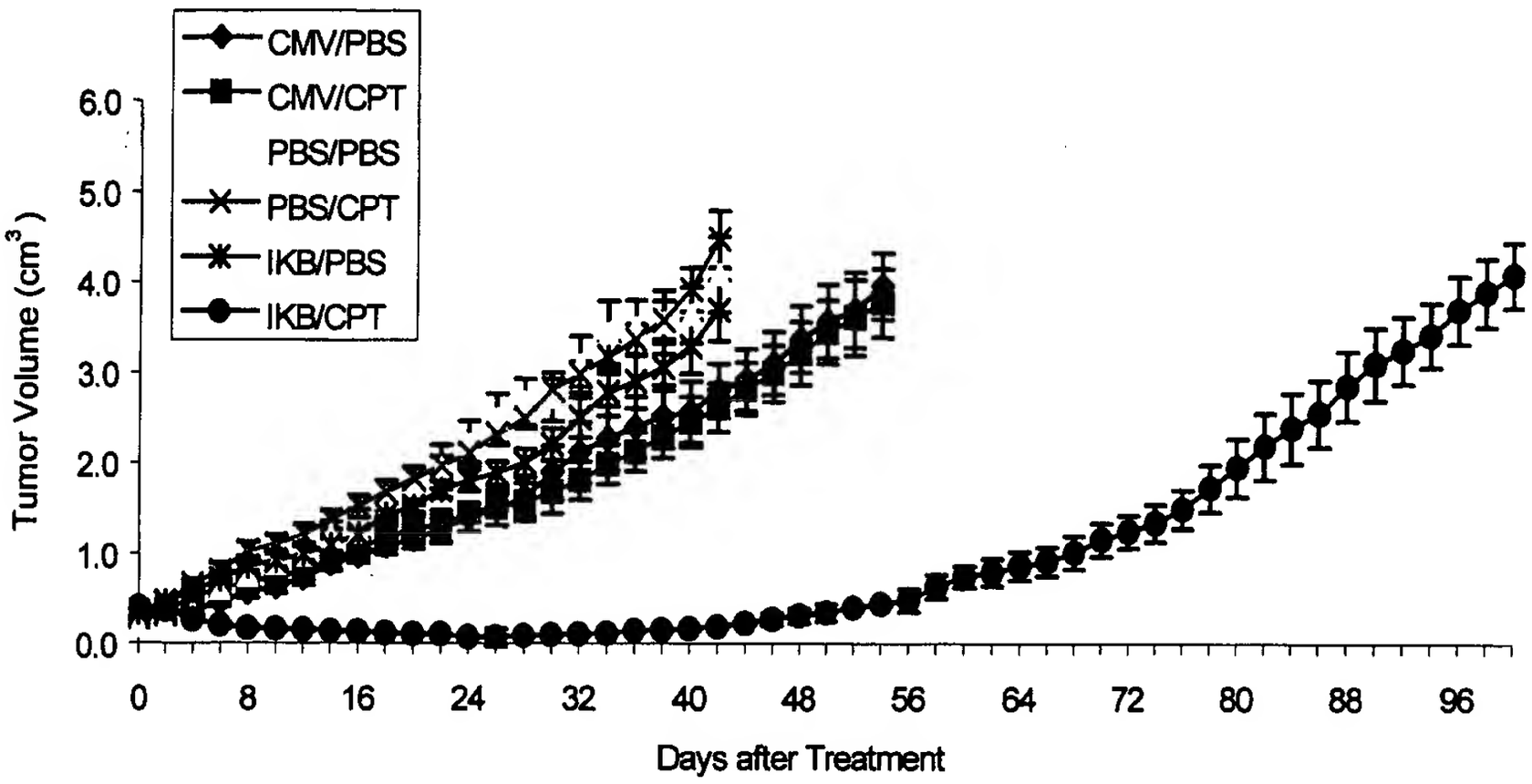
3.00E+05
2.00E+05
1.00E+05
0.00E+00
Cells per Well
1000ng
500ng
100ng
LOVO

Cusack, JC

Fig 1B (copy)

3A


CMV/PBS
CMV/CPT
PBS/PBS
PBS/CPT
IKB/PBS
IKB/CPT
Tumor Volume (cm3)
6.0
5.0
4.0
3.0
2.0
1.0
0.0
0
8
16
24
32
40
48
56
64
72
80
88
96
Days after Treatment

3B

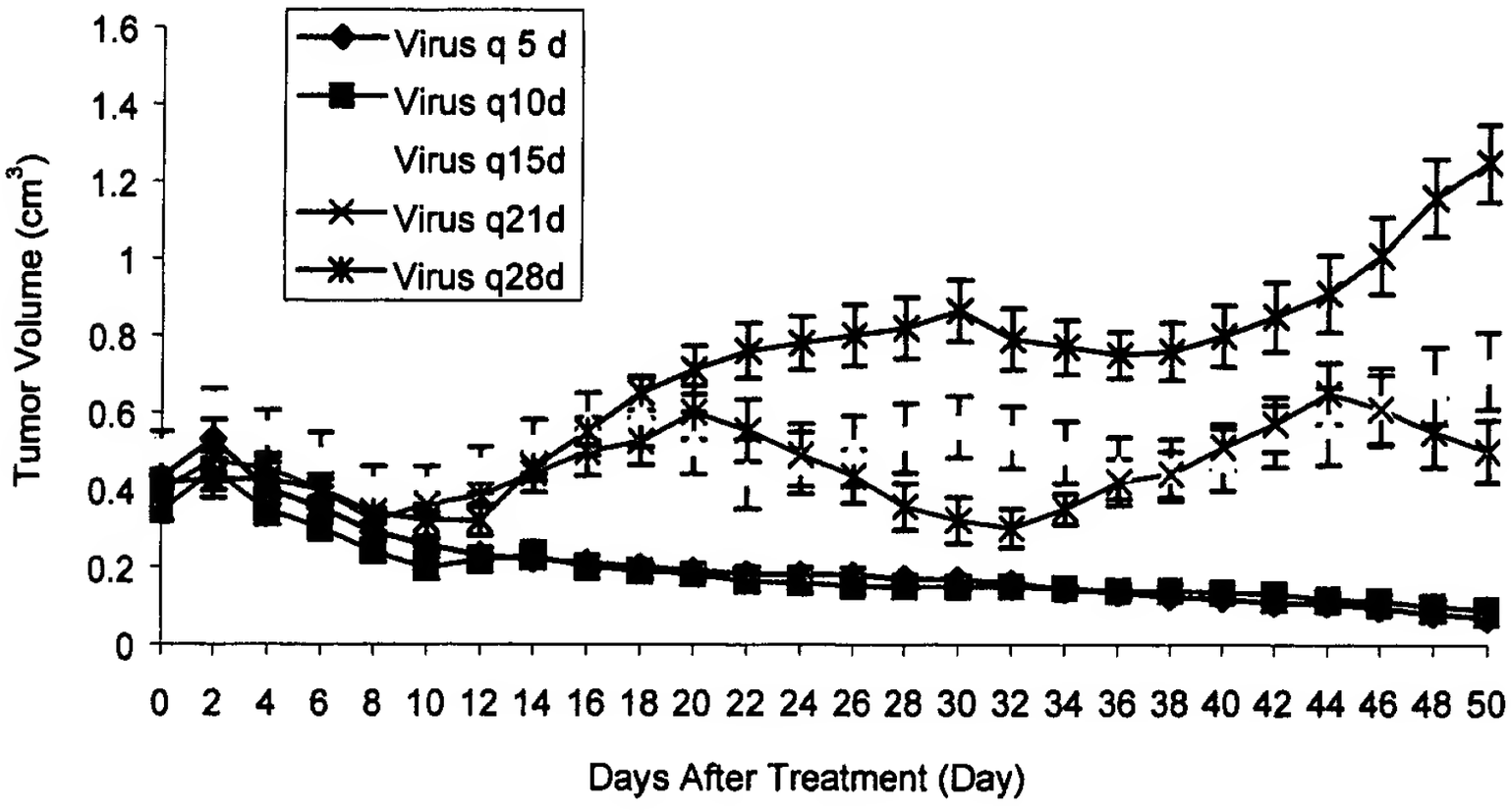

Virus q 5 d
Virus q10d
Virus q15d
Virus q21d
Virus q28d
Tumor Volume (cm3)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44 46 48 50
Days After Treatment (Day)

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Fig 2A+2B

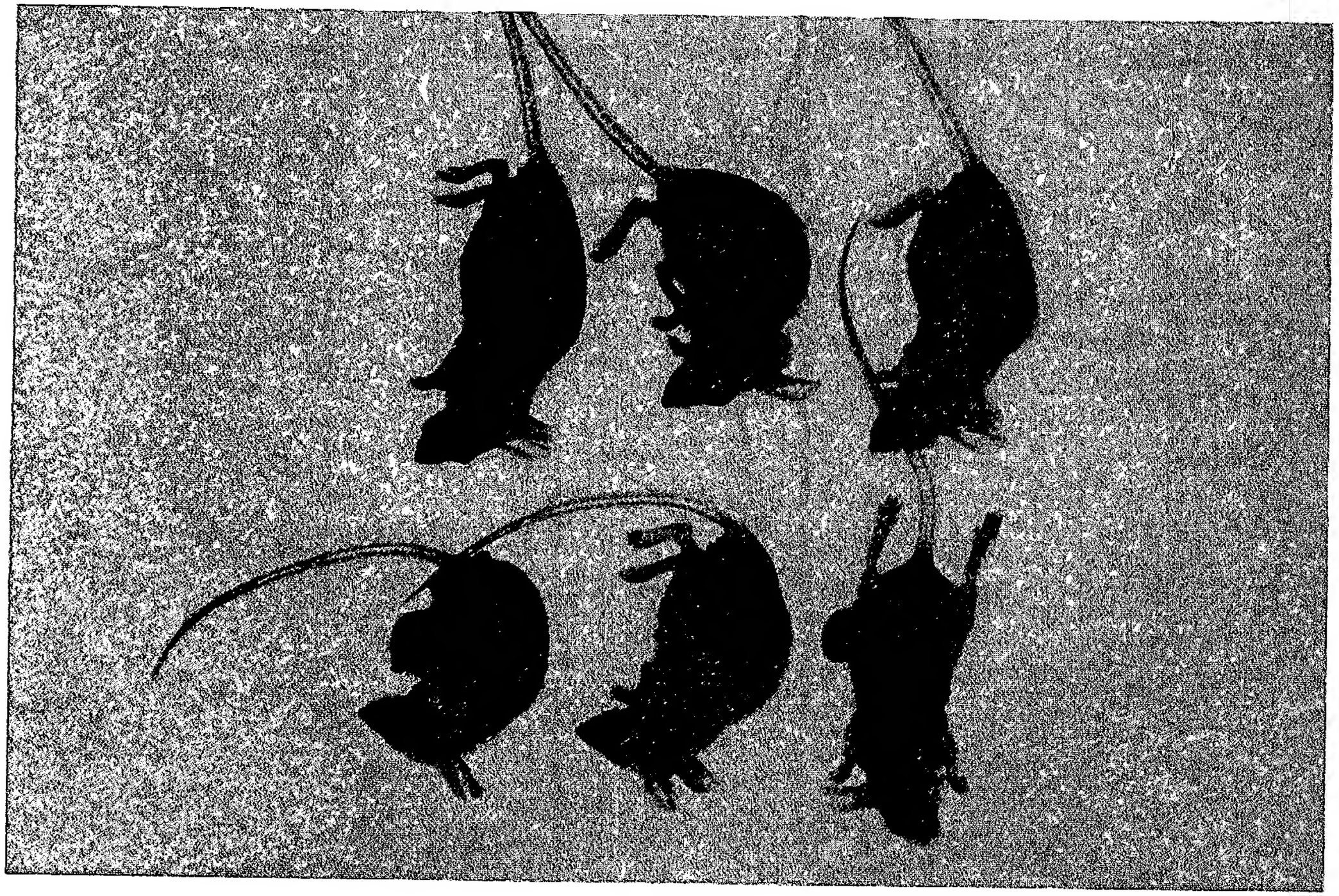

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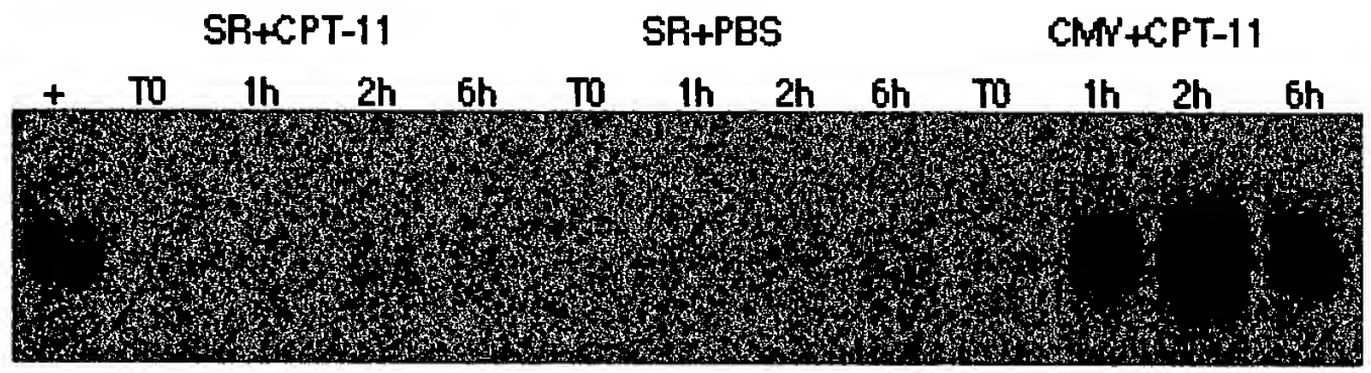
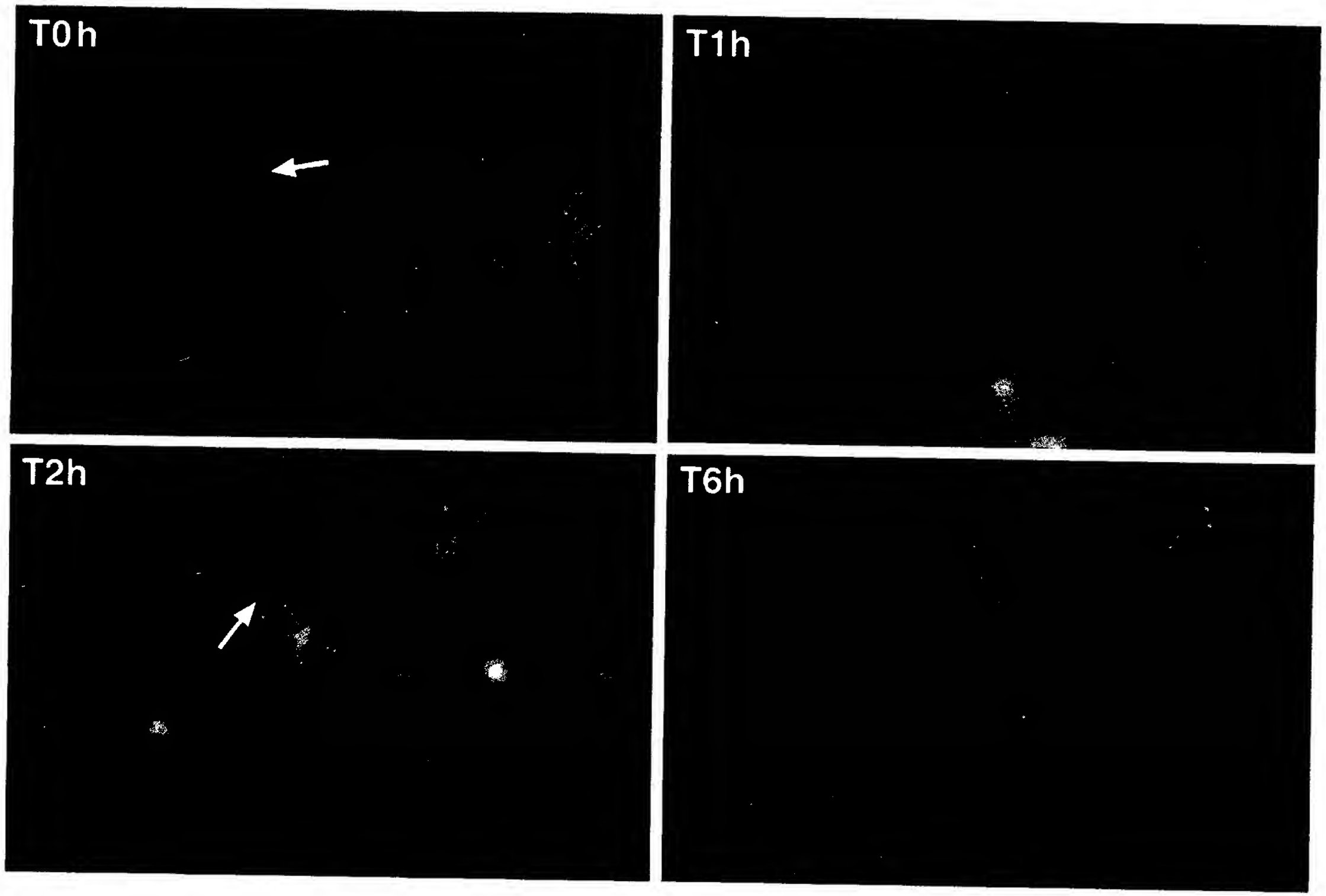
Fig 2C (copy)


SR+CPT-11
SR+PBS
CMV+CPT-11
+
T0
1h
2h
6h
T0
1h
2h
6h
T0
1h
2h
6h

Cusack, JC

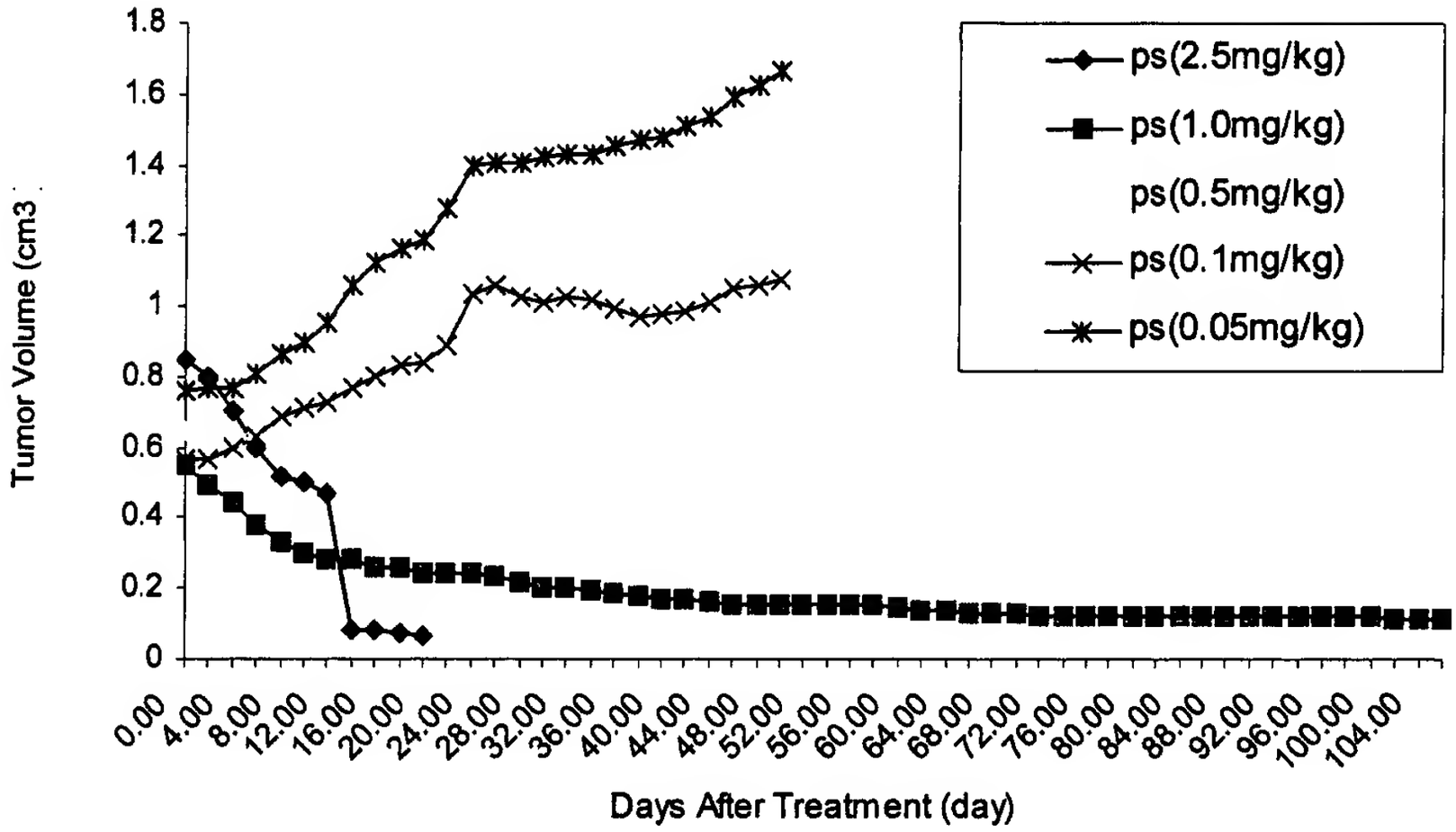
Fig 3 (copy)


T0h
T1h
T2h
T6h

CUSACK JC
FIG 4 (copy)

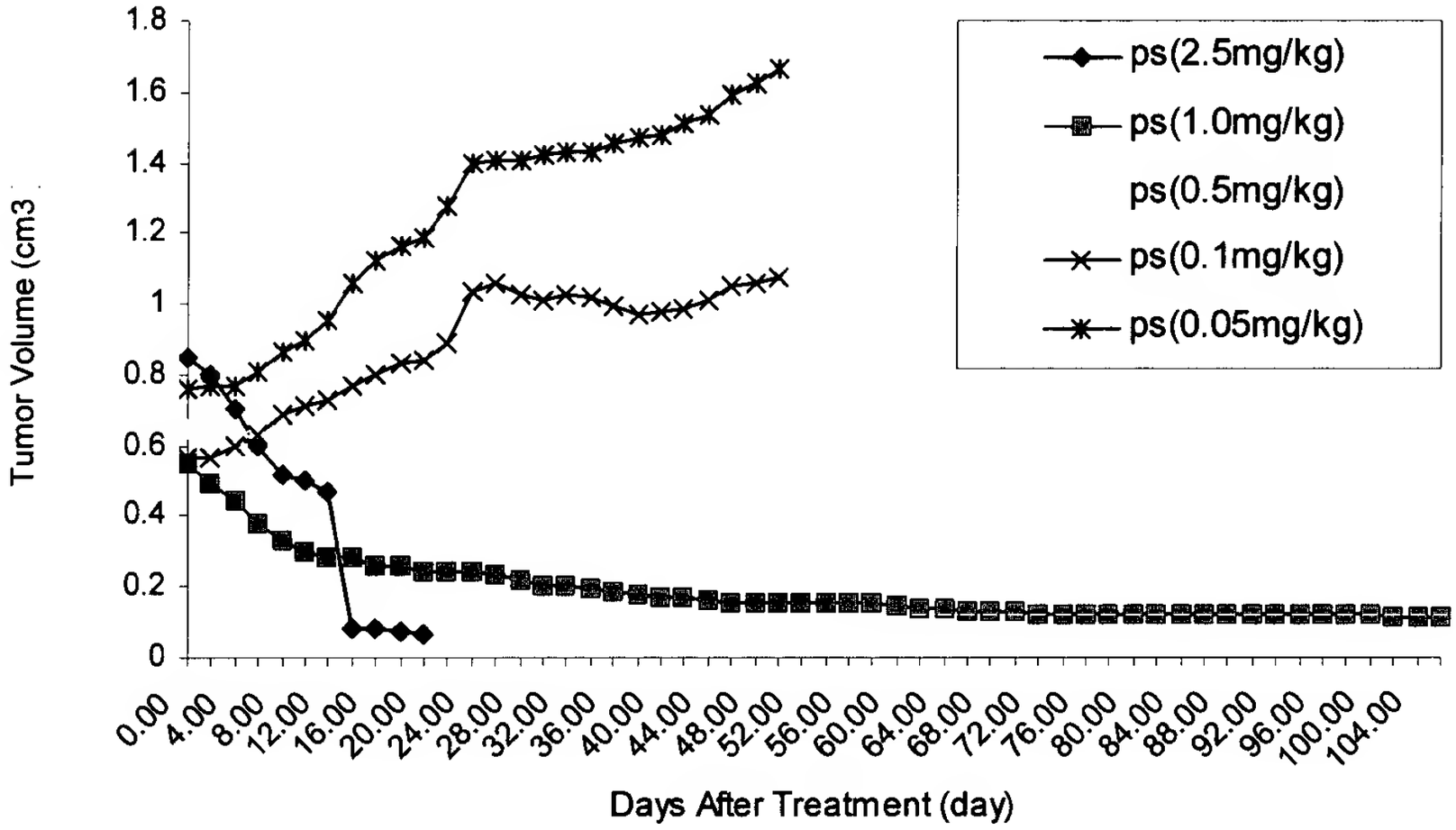
Induction of tumor regression by combined treatment with an NF-κB inhibitor (PS-341) and with chemotherapy (CPT-11). LOVO colorectal tumors were formed in mice and the animals were given chemotherapy plus PS-341 systemically every five days over the indicated time period. PS-341 (ps) was varied for the concentration given (as shown on the figure). The tumor response varied according to the dose given, with the highest dose (2.5 mg/kg) leading to virtually complete tumor elimination. No lethality was observed in the mice even at the highest treatment level.

Response of LOVO Tumors to Combined Treatment with CPT-11 and Increasing Dosage of PS-341



Induction of tumor regression by combined treatment with an NF-κB inhibitor (PS-341) and with chemotherapy (CPT-11). LOVO colorectal tumors were formed in mice and the animals were given chemotherapy plus PS-341 systemically every five days over the indicated time period. PS-341 (ps) was varied for the concentration given (as shown on the figure). The tumor response varied according to the dose given, with the highest dose (2.5 mg/kg) leading to virtually complete tumor elimination. No lethality was observed in the mice even at the highest treatment level.

Response of LOVO Tumors to Combined Treatment with CPT-11 and Increasing Dosage of PS-341



In review, Cancer Research.

Inducible Chemoresistance to CPT-11 in Colorectal Cancer Cells and a Xenograft Model is Overcome by Inhibition of NF-κB Activation

James C. Cusack, Jr.,[1,2] Rong Liu, Albert S. Baldwin, Jr.[1,2]

Running Title: Inhibition of NF-κB activation enhances response to CPT-11.

Key words: Super-repressor IκBα, NF-κB, camptothecin resistance, colorectal cancer, CPT-11.

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ABSTRACT

Limited studies have indicated that some chemotherapies activate the transcription factor NF-κB and that this leads to suppression of the apoptotic potential of the chemotherapy. In contrast, it was reported recently that stable inhibition of NF-κB in four different cancer cell lines did not lead to augmentation of the chemotherapy-induced apoptosis. In this study, we have focused on colorectal cancer, which is known to be highly resistant to genotoxic chemotherapy and gamma irradiation. First, we show that the topoisomerase I inhibitor CPT-11 activates NF-κB in most colorectal cancer cell lines. We then examine a therapeutic strategy that utilizes adenovirus-mediated transfer of the super-repressor IκBα to inhibit NF-κB activation as an adjuvant approach to promote chemosensitivity in colorectal tumor cells to treatment with CPT-11. These data demonstrate that the protection from apoptosis induced in response to CPT-11 treatment is effectively inhibited by the transient inhibition of NF-κB in a variety of human colon cancer cell lines and in a tumor xenograft model resulting in a significantly enhanced tumoricidal response to CPT-11 via increased induction of apoptosis. These findings indicate that the activation of NF-κB by chemotherapy is an important underlying mechanism of inducible chemoresistance.

INTRODUCTION

The transcription factor NF-κB (1) has been shown to regulate apoptosis in several different settings (see Wang et al. 1998 for a review) (2). Importantly, several groups have shown that NF-κB is activated by exposure of cells to chemotherapy (see Wang et al., 1999 for a review) (3). Recently, we studied the human fibrosarcoma cell line HT1080 and showed that the activation of NF-κB by the chemotherapy daunorubicin strongly suppressed the apoptotic potential of this chemotherapy (4). Furthermore, tumors derived from HT1080 cells were induced to undergo apoptosis and regression when animals were treated with the topoisomerase I inhibitor CPT-11 in parallel with adenoviral delivery of a modified form of IκBα, the natural inhibitor of NF-κB. These data argued that the activation of NF-κB by chemotherapy provides an important mechanism for inducible chemoresistance. In apparent contrast with these studies, it was reported recently (5) that four different cell lines that stably express the modified form of IκBα did not show enhanced cytotoxicity in response to chemotherapy even though the chemotherapies activated NF-κB. We report here continued studies on the role of NF-κB in controlling inducible chemoresistance and we report a therapeutic regimen involving the inhibition of NF-κB which leads to dramatic anti-tumor responses. Additionally, we show that the stable inhibition of NF-κB via IκB expression is not a consistent experimental approach to test the role of NF-κB in chemoresistance.

In order to extend our original studies, we have focused on colorectal cancers. Like most solid tumors, colorectal cancers are frequently resistant to chemotherapy and irradiation (6). Several of the cellular mechanisms that determine sensitivity of cancer cells to genotoxic therapies have recently been elucidated (7-10). For example, one mechanism for chemoresistance

is the upregulation of the multidrug resistance gene product (MDR1), which is responsible for pumping chemotherapy agents from the cells (11). Other mechanisms of resistance appear to affect a cancer cell's ability to undergo apoptosis, the major mechanism by which chemotherapy and radiation induce the killing of tumor cells (12-14). Thus mutation in the p53 tumor suppressor gene leads to acquired resistance that impairs p53-dependent responses to apoptotic stimuli, and may promote cancer cell survival and proliferation in some cancer cells (15). Another important mechanism for resistance to chemotherapy is inducible chemoresistance, a process whereby exposure of tumor cells to cancer therapy leads to their resistance to apoptosis (11). As described above, we have proposed that a major mechanism for inducible chemoresistance is the upregulation of the transcription factor NF-κB.

The objectives of this paper are to determine if the principle of reversing inducible chemotherapy resistance, as a means of enhancing the apoptotic response to chemotherapy treatment, can be applied broadly to the treatment of colorectal cancer in preclinical models. Specifically, these experiments explore whether the topoisomerase I inhibitor CPT-11, a chemotherapy showing promise for cancers of different tissue origin (6, 16-20), induces activation of NF-κB in a variety of human colorectal cancer cells. We also examine whether transient inhibition of NF-κB activation concurrent with CPT-11 exposure enhances the anti-cancer effect of CPT-11 among different colorectal cancer cell lines, and several of the cell lines used by Bentires-Alj et al. (5). Finally, in an effort to determine the optimal dosing schedule necessary to maximize tumoricidal response in a colon cancer xenograft model, we examine a variety of treatment schedules that combine inhibition of NF-κB activation with CPT-11 administration. It is hoped that the results of these studies will contribute to the design and

implementation of a novel therapeutic approach that improves patients' response to systemic treatment for metastatic colorectal cancer as well as other forms of cancer.

MATERIALS AND METHODS

Adenovirus Vectors. The recombinant adenovirus vectors used in this study were replication-defective Ad5-based vectors constructed with the transgene expression driven by the CMV early/intermediate promoter/enhancer. All vectors were expanded in 293 cells and purified and titered as previously described (21). The vector Ad.CMV.IκBα expresses the super-repressor form of IκBα that is mutated at serine residues 32 and 36, and functions as a potent and specific repressor of NF-κB-mediated events (4, 22). The control vector Ad.CMV3, generously provided by J. A. Roth (U. T. M. D. Anderson Cancer Center, Houston, TX), contains a CMV promoter similar to Ad.CMV.IκBα, but lacks a transgene insert.

Chemotherapy Agents. Camptothecin is a specific inhibitor of mammalian DNA topoisomerase I. The camptothecin analogue CPT-11 [7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxycamptothecin] and its active metabolite SN38 (7-ethyl-10-hydroxycamptothecin) were generously provided by J. Malczyn, Pharmacia and Upjohn Co. (Kalamazoo, MI).

Cell Culture. The human colon cancer cell line LOVO was obtained from American Type Culture Collection (ATCC, Rockville, MD). The LOVO cells were grown in F-12 (Ham) with 20% fetal bovine serum (FBS). The colon cancer cell lines SW1463, SW837, SW620, SW480

were obtained from ATCC and grown in L-15 with 10% FBS. The colon cancer cell lines KM12-L4 and KM12-SM (generous gifts of J. Fidler, Houston, TX), and HT-29, WiDR and CL188 (obtained from ATCC) were grown in MEM with 10% FBS. The colon cancer cell line CCD841 (ATCC) was grown in DMEM with 10% FBS, and NCI H508 was grown in RPMI with 10% fetal bovine serum. All media was obtained from Life Technologies, Inc. (Gaithersburg, MD), and supplemented with 100μg/ml penicillin G, and 100 μg/ml streptomycin. MCF-7 and HCT 116 were obtained from ATCC and grown in EMEM with 10% FBS and McCoy's 5A medium with 10% FBS respectively. Cell cultures were maintained at 37°C.

Cell Growth Inhibition. Human cancer cells (5 - 8 X 10^4) were seeded in 6-well plates and infected with Ad.CMV.IκBα at a multiplicity of infection (MOI) of 20 per target cell when cells reached 20 percent confluence. The control adenovirus vector Ad.CMV3 was used to infect the control group. Drug treatment with SN38, the active metabolite of CPT-11, was administered 24 h after virus infection at a final concentration of 1000ng/ml, 500ng/ml or 100ng/ml. Daily cell counts were performed for 4 days. Experiments were performed in triplicate.

NF-κB Activation Assay. Activation of NF-κB in response to treatment with chemotherapy was determined by the Electrophoretic Mobility Shift Assay (EMSA) as previously described (23). For *in vitro* experiments, cancer cells were cultured in 100mm dishes until 50~70% confluence was achieved. Cells were infected with Ad.CMV.IκBα (MOI = 100) for 1 h then washed with PBS and refed media. Cells were treated with SN38 (1μg/ml) 24 h after adenovirus infection. Cells were then harvested at time 0, 1, 2, and 6 h following treatment with SN38.

Nuclear extracts were prepared by collecting cells, then washing and suspending them in hypotonic buffer. The nuclear pellet was separated by centrifugation, and the cytoplasmic supernatant was discarded. The nuclei were then resuspended in a low-salt buffer to high-salt buffer, and the soluble protein was released by centrifugation, collected, and stored at –70°C. The DNA probe used contains an NF-κB site (underlined) from the *H-2κ^b* gene (5'-CAGGGCTGGGGATTCCCATCTCCACAGTTTCACTTC-3') (3). In brief, 7 μg of nuclear extracts were preincubated with 1 μg of poly(dI-dc) in binding buffer (10 mM Tris, 50 mM NaCl, 20% glycerol, 0.5 mM EDTA, 1 mM DTT) for 10 min at room temperature. Approximately 20,000 cpm of ^{32}P-labeled DNA probe was then added and allowed to bind for 15 min. The complexes were then separated on a 5 % polyacrylamide gel and autoradiographed.

The procedure for obtaining nuclear extracts from cancer cells was modified for tumor samples as follows: subcutaneous tumors were harvested following treatment, and snap-frozen in liquid nitrogen. Frozen tumor tissue were morselized over liquid nitrogen and scraped into 10 ml conical tubes containing 5 ml of solution A (0.3M sucrose, 60mM KCl, 15mM NaCl, 15mM Hepes pH 7.5, 2mM EDTA, 0.5mM EGTA, 14mM 2-ME, 0.1% NP40). The mixture was ground into a slurry, and the nuclear solution was layered. On the top of the slurry, 2.5 ml of Solution B (0.88M sucrose, 60mM KCl, 15mM NaCl, 15mM Hepes pH 7.5, 2mM EDTA, 0.5mM EGTA, 14mM 2-Me) was added and the mixture was spun down for 10 min at 3000 RPM. The pellet was brought up to 1 ml Solution D (1M sucrose, 15 mM KCl, 15 mM NaCl, 15 mM Hepes pH 7.5, 0.1 mM EDTA, 0.1 mM EGTA, 14 mM 2-ME), and layered on top of 2.5 ml of Solution D, then spun down at 3000 RPM for 10 min. The supernatant was aspirated, and the pellet was resuspended in NE buffer (20mM Tris pH 8.0, 120 mM NaCl, 1.5mM MgCl, 0.2mM EDTA,

0.5mM PMSF, 25% Glycerol) with proteinase inhibitor (Leupeptin 10μg/ml, Aprotinin 25μg/ml, Pepstatin 5μg/ml). The mixture was incubated on ice for 10 min, then spun down at full speed for 15 min at 4°C. The nuclear protein remaining in solution was then collected.

***In Vivo* Evaluation of Tumor Growth.** The ability of the super-repressor IκBα to enhance sensitivity to CPT-11 was assessed in a LOVO xenograft model. The tumors were established by injecting 5 X 10^6 LOVO cells subcutaneously into the flank of nude mice (NCR nu/nu athmic nude mice, 5-6 wks, female and weight of 18-20 g) (Taconic Germantown, NY). Once tumors reached a mean diameter of 8-10mm, treatment was initiated. Animals were treated on day 0, day 7, and day 14 with a single-pass intratumoral injection of adenovirus expressing the super-repressor IκBα (Ad.CMV.IκBα at a concentration of 1 X 10^{10} pfu/200μl), control adenovirus Ad.CMV3 (1 X 10^{10} pfu/200μl), or vehicle alone using a previously described technique (21). Based on previous published reports of tumoricidal response to CPT-11 in xenograft models (24, 25), a dosage of 33mg/kg every 4 days was selected and administered i.v. via tail-vein injection during the 20-day treatment period. A total of 5 drug treatments was administered. PBS was administered i.v. as a control vehicle. Tumor diameter along two orthogonal axes was recorded every other day until tumors approached 20mm mean diameter at which point animals were sacrificed according to protocol. Tumor volume was calculated by assuming a spherical shape of the tumor, using the formula: volume = 4/3 πr^3 where r is ½ (mean diameter of the tumor).

The effect of differing schedules of administration of Ad.CMV.IκBα on tumoricidal response was assessed in a xenograft model. Nude mice bearing subcutaneous LOVO tumors (mean diameter 8-10mm) were treated over a 50 day period with intravenous CPT-11 (33mg/kg every 4

days) and differing schedules (every 5d, 10d, 15d, 28d) of intratumoral injection of the adenovirus expressing the super-repressor IκBα (Ad.CMV.IκBα at a dosage of 1 X 10^{10} pfu/200μl).

Assays to Detect Apoptosis and Transgene Expression *In Vivo*. LOVO tumor xenografts (1-cm diameter) were treated with a single intratumoral injection of Ad.CMV.IκBα (1 X 10^{10} pfu/200μl). Twenty-four h after virus administration, CPT-11 (33mg/kg) was given i.v. by tail-vein injection. Tumors were harvested at time 0, 1h, 2h, and 6h after drug treatment, embedded in O.C.T. mounting medium (VWR, Sakura Finetec U.S.A. Inc., Torrance, CA Lot # 1179027), and snap-frozen in 2-methylbutane (Aldrich Chemical Co., Milwaukee, WI) over liquid nitrogen, then stored at -70°C. Four μm thick sections were cut and collected on charged and precleaned microscope slides (Fisher Scientific, Pittsburgh, PA). Cryosections were stained using a double immunohistological staining technique to detect the presence of exogenous IκBα in tumor cells and to determine the level of apoptosis, determined by the deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay in tumors. Exogenous IκBα was detected using a murine antibody (Anti-HA monoclonal antibody at the dilution of 1/200) to the hemagglutinin (HA) tag (fluorescent red) present on the exogenous IκBα expressed by the Ad.CMV.IκBα. The reaction mixture was fixed with 10% neutral buffered formalin and blocked with 2% BSA followed by incubation for 1 h. Binding was detected with a 1:100 dilution of Rhodamine-congugated goat anti-mouse secondary antibody. Apoptosis was detected using the In Situ Cell Death Kit (Boehringer Mannheim Corporation, Indianapolis, IN) and a fluorescent green

antibody to detect TUNEL positive cells (arrows). Analysis of tumor sections was performed using a 2-color fluorescent microscope at 100X magnification.

RESULTS

NF-κB activation is induced by SN-38 in most colorectal cancer cell lines. Previously we have analyzed the ability of daunorubicin and the topoisomerase I inhibitor CPT-11/SN38 to activate NF-κB in fibrosarcoma cells (3, 4). While others have reported that different chemotherapy agents activate NF-κB in different cell types, a recent report suggested that stable inhibition of NF-κB did not lead to enhanced cytotoxicity in several cancer cell lines, including the colorectal cancer cell line HCT116 and the breast cancer cell line MCF-7 (5). We explored the ability of SN38, the active metabolite of CPT-11, to activate NF-κB in a panel of colorectal cancer cell lines plus MCF-7 cells. In order to inhibit NF-κB we used transient inhibition via the adenoviral expression of the super-repressor IκBα. Our data from electrophoretic mobility shift assays (EMSAs) demonstrated that NF-κB activation, as measured by nuclear translocation of NF-κB, is induced at 1 h after SN38 treatment and persisted up to 6 h in 10 out of 11 cancer cell lines tested and in MCF-7 cells (Fig. 1A). One of the cell lines, HT-29, did not show activation of NF-κB in response to SN38 treatment. The serine to alanine mutations at serine residues 32 and 36 of the super-repressor form of IκBα inhibits signal-induced phosphorylation and subsequent proteasome-mediated degradation of IκBα. Cytoplasmic binding of the mutant IκBα protein to the nuclear localization sequences of NF-κB thereby blocks nuclear translocation and subsequent binding of NF-κB to κB binding sites on DNA, effectively blocking NF-κB-mediated transcription (4). We have previously shown *in vitro* that pretreatment of LOVO cells with

Ad.CMV.IκBα prior to SN38 administration markedly decreased nuclear translocation of NF-κB in response to SN38 (26). We show here that adenoviral delivery of the super-repressor IκB, but not control adenoviral infection, suppressed NF-κB activation (Fig. 1A).

The activation of NF-κB inhibits SN38-induced cytotoxicity. We determined whether inhibition of NF-κB activation by SN38 would augment the cytotoxic response of this chemotherapy. We analyzed cell numbers for 6 of the colorectal cancer cell lines and for MCF-7 breast cancer cells following SN38 treatment in the presence or absence of adenovirus encoding the super-repressor form of IκBα. The control was to include an adenovirus without IκBα cDNA. The data clearly show (Fig. 1B) that SN38 is cytotoxic in a dose-dependent manner and that inhibition of NF-κB augments the cytotoxic response. Previously, Bentires-Alj et al. established several cancer cell lines, including HCT116 and MCF-7, stably expressing super-repressor IκBα and found that cytotoxicity was not enhanced when NF-κB was inhibited (5). Our data contradict those conclusions, at least in two of these cell lines, since we show that transient inhibition of NF-κB by adenovirus expression of the super-repressor IκBα strongly augments the cytotoxic response to SN38 (see Discussion). Furthermore, since the response is seen in both p53 mutated (KM12L4, KM12SM, WiDR) and p53 wild-type (CCD841, MCF-7, HCT116, LOVO) cell lines, our data suggests that the enhancement of cytotoxicity induced by inhibition of NF-κB is independent of p53 function (see Discussion).

Sensitivity of colorectal tumors to CPT-11 is markedly enhanced by NF-κB inhibition. Experiments were performed to determine if the super-repressor IκBα could promote enhanced

sensitivity of LOVO xenograft tumors to CPT-11 (Fig. 2A). As previously described (3), tumor growth following combined treatment with super-repressor IκBα and CPT-11 was significantly less than treatment with the super-repressor IκBα or CPT-11 alone ($p < 0.0001$, analyzed by ANOVA). Delivery of IκB once a week for 3 weeks combined with CPT-11 treatment twice a week for three weeks lead to dramatic inhibition of tumor growth (Fig. 2A). Interestingly, tumor size increased after 50 days and ultimately achieved a rapid growth rate. However, modification of the treatment schedule to treat tumors every 5 or 10 d in combination with CPT-11 administration over a 50 day period resulted in a persistent tumoricidal response (Fig. 2B), and ultimately long-term cures following cessation of treatment at day 50. Tumor growth in groups receiving treatment with Ad.CMV.IκBα every 5 or 10 d was significantly less compared to groups receiving viral treatment every 15, 21 or 28 d ($p < 0.0001$, analyzed by ANOVA). Tumor regrowth in the groups treated with virus every 15d, 21d, and 28d, suggests that IκBα played an important role in the enhancement of the tumoricidal response to CPT-11. Long-term follow-up of treatment groups following cessation of both viral and CPT-11 treatment on day 50 demonstrated between fifty and sixty-six percent of animals receiving viral treatment every 5 or 10 d remained tumor-free 5 months after discontinuation of all treatments (Fig. 2C).

Super-repressor IκBα inhibits nuclear translocation of NF-κB induced by CPT-11 *in vivo*. It was important to show that CPT-11 actually induced NF-κB in tumors and that the adenoviral delivery of IκBα into tumors successfully inhibited NF-κB activation. EMSA assay for nuclear protein obtained from tumor tissue extracts demonstrated that NF-κB activation was induced by CPT-11 at 1 h after systemic drug administration with a peak activation of NF-κB

observed at 2h (Fig. 3). NF-κB activation in tumors was blocked by pretreatment with the adenovirus expressing the super-repressor IκBα. The results demonstrate that *in vivo* activation of NF-κB following treatment with CPT-11 was inhibited by the expression of the super-repressor IκBα but not the control vector.

Apoptosis is rapidly induced by CPT-11 following pretreatment with the NF-κB super-repressor IκBα. We measured viral infectivity and IκBα expression along with the induction of apoptosis in LOVO tumors following systemic CPT-11 treatment. Two-color immunohistochemical staining of tumors injected with the adenovirus expressing the super-repressor IκBα and treated with CPT-11 demonstrated HA-positive cells diffusely throughout the tumor at all time points sampled, indicating successful adenovirus-mediated transfer of the super-repressor IκBα gene to tumor cells (Fig. 4). Staining for TUNEL-positive cells demonstrated that only a few tumor cells were undergoing apoptosis prior to CPT-11 treatment and at one h following treatment. In comparison, increasing levels of apoptosis were observed at both 2 and 6 h after drug-treatment. Importantly, treatment with the control virus Ad.CMV3 and CPT-11 or with Ad.CMV.IκBα and PBS induced apoptosis in less than 1% of tumor cells at all time points sampled (data not shown). These data demonstrate that the activation of NF-κB in colorectal tumors suppresses the apoptotic potential of the chemotherapeutic response.

DISCUSSION

NF-κB activation induced by cancer therapies blocks the induction of apoptosis. Most cancer therapeutics function by killing cells through the induction of the apoptotic pathway. In fact, resistance to the induction of the apoptotic response is a principle mechanism by which cancer cells protect against cell killing (12). We have previously reported that the activation of NF-κB by TNF-α, ionizing radiation, and the cancer chemotherapeutic compound daunorubicin leads to an inhibition of the apoptotic response induced by these stimuli in fibrosarcoma cells (4). Similar results were obtained by others (27-29) relative to TNF-α. Thus, we and others have proposed that potential apoptotic stimuli initiate two distinct signaling pathways: one that leads to activation of apoptosis and one that leads to NF-κB activation, which induces a cell survival response through the inhibition of apoptosis (2, 4, 30-34). Contrasting findings such as those reported by Kasibhatla et al. (35), in which stress-induced expression of FasL (leading to Fas-mediated apoptosis) in human leukemic Jurkat cells required NF-κB activation, has led some authors to conclude that NF-κB serves multiple functional roles under different conditions (36). The mechanism by which chemotherapy activates NF-κB is presently unknown and is the focus of ongoing investigation in our laboratory. However, the mechanism whereby NF-κB suppresses apoptosis is better understood and involves the induction of expression of genes which block the caspase cascade (2). We have previously shown that inhibition of inducible NF-κB activation by transient expression of the super-repressor IκBα leads to a dramatic improvement in the killing response of tumor cells when exposed to apoptotic stimuli (3). Based on these studies, we conclude that the apoptotic response to conventional chemotherapy and irradiation may be augmented by the inhibition of NF-κB activation in resistant cancer cells. The objective of this

paper was to evaluate the role of transient inhibition of NF-κB as an adjunct to the topoisomerase I inhibitor CPT-11 for the treatment of colorectal cancer cells.

Use of CPT-11 in the treatment of colorectal cancer. Colorectal cancer is the second most common cause of mortality from malignancy in the U.S. accounting for approximately 57,000 deaths in the U.S. in 1997 (37). Approximately 50% of these patients will eventually die of metastatic disease (38). The relatively high mortality rate of patients who are not cured by surgical treatment results from the resistance that most cancers have to conventional chemo- and radiation therapies (39-41). Recently efforts to overcome the 70-80% rate of resistance of colorectal cancers to conventional therapies have directed the use of new compounds with reported higher levels of sensitivity in clinical trials. CPT-11 is one such promising agent used to treat a variety of solid tumors including colorectal cancer and lung cancer (6, 17-20, 42). *In vitro* studies have demonstrated CPT-11 to have sustained activity against chemotherapy-resistant colon cancer cell lines, including those having the multi-drug resistance (MDR) phenotype (6). In addition, results from clinical trials indicate CPT-11 to be a promising anticancer agent, used as a single agent or in combination with other agents, with a duration of response from several months to 1 year (17-20, 43). Owing to its lack of cross-resistance with 5-fluorouracil (5-FU), promising clinical responses suggests CPT-11 may be an effective second-line agent in the treatment of patients who have failed first-line treatment with 5-FU based regimens (6). A recently completed multicenter randomized clinical trial found that CPT-11 increased the one-year overall survival rate 2.6 times greater than supportive care in patients who had failed conventional treatment with 5-FU (16).

SN38 induces activation of NF-κB in a variety of human colorectal cancer cell lines. In our previous studies we have demonstrated that NF-κB activation may result from exposure of cancer cells to a variety of apoptotic stimuli including TNF-α, chemotherapy and irradiation (4). In this report we have evaluated a wide variety of human colorectal cancer cells to determine if this inducible response is widely observed or incidental. To evaluate this response, we have selected a variety of resistant colorectal cancer cell lines including those that are mutated for the p53 gene (WiDR, KM12L4, KM12SM, SW480, SW620), contain the K-ras oncogene (LOVO, HCT116, SW480, SW620) as well as those that overexpress Bcl-2 (KM12L4, SW480). The enhanced cytotoxicity responses attained through inhibition of NF-κB were found to be independent of the status of p53, K-ras or Bcl-2 expression. These results do not imply that expression of these proteins is not relevant to cancer therapy, but that the enhancement of cytotoxicity can be attained in their absence of expression. In preliminary studies, anticancer agents that traditionally have been used to treat patients with metastatic colorectal cancer, including 5-fluorouracil (5-FU) and mitomycin C (MMC), were found to only weakly induce NF-κB activation in a variety of colorectal cancer cell lines tested (data not shown). In some cell lines no activation of NF-κB was observed following treatment with these agents. In contrast, SN38, the active metabolite of CPT-11, was found to activate NF-κB in 10 out of 11 colorectal cancer cell lines tested. Importantly, in all cases in which NF-κB activation was induced by SN38, inhibition of activation was facilitated by pretreatment with super-repressor IκBα but not the control vector. These findings are consistent with our observations in a variety of different cancer subtypes including pancreatic cancer, sarcoma, and breast cancer in which SN38 was

found to be a very potent and consistent inducer of NF-κB activation (data not shown). In addition, relative to a variety of different anticancer agents we have tested, the level of inducible NF-κB activation following treatment with SN38 is surpassed only by TNF-α (data not shown).

Inhibition of SN38-induced NF-κB activation enhances sensitivity to SN38 in a variety of resistant colorectal cancer cell lines. To evaluate the role of SN38-induced NF-κB activation on the chemosensitivity of a variety of resistant colon cancer cell lines, we pretreated these cells with the super-repressor IκBα. In all cell lines tested, sensitivity to SN38 was markedly enhanced by transient inhibition of NF-κB activation. Furthermore, enhanced chemosensitivity was observed at all concentrations of SN38 tested. This has important clinical relevance, due to the inability to achieve the requisite therapeutic dosages of chemotherapy in patients, due to dose-limiting toxicity. At the lower tolerated levels of chemotherapy attained in patients, we would predict based on these findings that enhanced chemosensitivity may be achieved by effective NF-κB inhibition in those cells. Although the ability to predict sensitivity of a patient's tumor to a specific chemotherapy agent is limited, the observed ability to augment the sensitivity of the variety of cell lines tested suggests that application of this combination therapy approach may have a broad impact on colorectal cancer patients receiving CPT-11.

Enhanced sensitivity to SN38 following inhibition of NF-κB activation was observed in cell lines that ranged in SN38 sensitivity from high (WiDR) to low (HCT116). The mechanism underlying chemotherapy resistance is likely multifactorial (44) as demonstrated in the wide range of genetic errors represented in the cell lines tested. Previous analysis of camptothecin (CPT) resistance in colorectal cancer cell lines from the NCI Anticancer Screen reported by

Goldwasser et al. (45) suggested that CPT uptake and expression of DNA topoisomerase I did not predict cytotoxicity in response to CPT, although the formation of cleavable complexes did reasonably predict CPT sensitivity. Our results suggest that inducible chemotherapy resistance, mediated by the transcription factor NF-κB, may also be a major determinant of sensitivity to CPT-11 and appears to be a shared survival mechanism among a wide variety of colon cancer cell lines. As described above, we observed enhanced chemosensitivity in both p53 mutated and wild-type cell lines, cells with and without oncogenic K-ras and independent of high or low levels of Bcl-2 expression.

Our findings using transient inhibition of NF-κB via adenovirus-mediated delivery of the super-repressor IκBα are in contrast to those recently reported by Bentires-Alj et al. (5), in which clones of HCT116 and the highly camptothecin-resistant breast cancer cell line MCF-7 were selected for stable expression of mutated (super-repressor) IκBα. Our data shown here and published previously suggest that the resistance to chemotherapy-induced apoptosis is mediated by the activation of NF-κB. However, in apparent contrast to our findings, in which inhibition of NF-κB activation in the parental cells lead to dramatically enhanced sensitization to SN38, the selected clones that were stably-transfected with mutated IκBα were not more sensitive to the various chemotherapy agents and TNFα, despite activation of NF-κB by these stimuli. This suggests that the process of selecting clones, that contain stable expression of mutated IκBα, leads to the acquisition of alternative survival mechanisms, necessary to overcome the NF-κB inhibition that occurs in the presence of constitutively-expressed, mutated IκBα. In contrast to the conclusions of the Bentires-Alj et al. report, our data suggest that, in fact, NF-κB *does* play a central role in inducible chemoresistance. Furthermore, our findings indicate that transient

inhibition of NF-κB activation is a potent adjuvant to the treatment of colon cancer with CPT-11. Additional studies are indicated to further evaluate the mechanisms of chemotherapy-induced NF-κB activation and the resulting anti-apoptotic response.

Dose intensification using serial administration of combined CPT-11 and NF-κB inhibition leads to complete tumor eradication in a colorectal cancer xenograft model. Application of combined NF-κB inhibition with CPT-11 for the treatment of human colorectal cancer appears to be most promising for chemotherapy resistant tumors. Cell lines such as WiDR, which were most sensitive to SN38, were found to have the minimum degree of enhanced response to NF-κB inhibition. Similarly, a minimal amount of enhanced sensitivity was observed *in vivo* in WiDR tumors that received combined treatment (data not shown). In contrast, the moderate resistance of LOVO to SN38 and CPT-11 *in vitro* and *in vivo* respectively, was dramatically overcome by inhibition of NF-κB activation. By inhibiting the inducible survival response that occurs with each individual drug treatment, through the activation of NF-κB, the maximum apoptotic response to CPT-11 was obtained. These results suggest that inhibition of NF-κB activation in combination with chemotherapy administration is a promising therapeutic strategy that warrants further evaluation in clinical trials. Furthermore, the results from these preclinical studies provide a rational basis for how to most effectively apply this therapeutic strategy in order to optimize the apoptotic response to CPT-11 in patients who are receiving treatment for metastatic colorectal cancer. In addition, current studies are underway to evaluate the potential role of small molecule inhibitors of NF-κB to enhance the apoptotic response to chemotherapy.

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LEGENDS

Fig. 1. A, the Electrophoresis Mobility Shift Assay (EMSA) was used to evaluate NF-κB activation induced by 1μg/ml SN38 in human colorectal and breast (MCF-7) cancer cell lines. Chemotherapy-induced activation of NF-κB was observed in 11 out of 12 cancer cell lines tested, suggesting that NF-κB activation is induced by SN38 in most colorectal cancer cell lines. Positive control (+) was KM12L4 cells treated for 2 h with 10 ng/ml TNFα (a potent activator of NF-κB). Figure shown is representative of the findings from two experiments. B, effect of NF-κB inhibition on human cancer cells treated with different concentrations of SN38. Cells were infected with adenovirus expressing the super-repressor IκBα (IκBα) or control virus (CMV) 24h prior to drug treatment. Cell counts obtained at 96 h following drug treatment are reported as the mean of triplicate cultures; *bars*, SD.

Fig. 2. The ability of the super-repressor IκBα to enhance sensitivity to CPT-11 was assessed in a LOVO xenograft model. A, Tumoricidal response of LOVO tumors to CPT-11 administered in combination with the adenovirus vector expressing the super-repressor IκBα (Ad.CMV.IκBα) compared to control adenovirus (Ad.CMV3), or vehicle alone. Adenovirus was administered as a weekly intratumoral injection of 1 X 10^{10} pfu/200μl of virus for three weeks. CPT-11 (33mg/kg) was administered i.v. every 4 days during the 20-day treatment period. Phosphate buffered saline (PBS) was administered i.v. as a control for treatment with CPT-11. Tumor diameter along two orthogonal axes was recorded every other day. Volume was calculated by assuming a spherical shape of the tumor, using the formula: volume = 4/3 πr^3 where r is ½ (mean diameter of the

tumor), and recorded as the mean +/- SE (bars) for each treatment group (n = 15-19). B, effect of differing schedules of Ad.CMV.IκBα administration on tumoricidal response was assessed *in vivo*. Nude mice bearing subcutaneous LOVO tumors (mean diameter 8-10mm) were treated over a 50 day period with intravenous CPT-11 (33mg/kg every 4 days) and differing schedules (every 5d, 10d, 15d, 28d) of intratumoral injection of the adenovirus expressing the super-repressor IκBα (Ad.CMV.IκBα at a dosage of 1 X 10^{10} pfu/200μl). Tumor volume was calculated by assuming a spherical shape of the tumor, using the formula: volume = 4/3 πr^3 where r is ½ (mean diameter of the tumor measured along 2 orthogonal axes). Tumor volume (y-axis) was recorded as the mean volume +/- SE (bars) for each treatment group (n = 10). C, representative animals from two treatment groups following 50 days of treatment as described in B. Pictured in the top row are mice that have received intratumoral treatments with Ad.CMV.IκBα administered every 15 days compared to adenovirus treatment every 5 days (bottom row).

Fig. 3. A, Electrophoresis Mobility Shift Assay (EMSA) of nuclear protein extracts from LOVO tumors following treatment with the super-repressor IκBα and CPT-11 was used to evaluate the ability of Ad.CMV.IκBα to inhibit NF-κB activation *in vivo*. Tumors were treated with a single intratumoral injection of Ad.CMV.IκBα (labeled IκBα above), or control vector Ad.CMV3 (labeled CMV above) as described in Materials and Methods. Tumors were treated with CPT-11 24 h after adenovirus injection, and harvested at time 0 (24 h after virus treatment), 1, 2, and 6 h after drug treatment. The positive control for this experiment was obtained from the colorectal cell line WiDr treated with SN38 at 2 h..

Fig. 4. Two color immunohistochemical staining of tumor sections was performed to evaluate adenovirus-mediated transfer of the super-repressor IκBα gene into tumor cells and the level of apoptosis induced by combined treatment of tumors with the super-repressor IκBα and CPT-11. Tumor cells expressing the HA-tagged super-repressor IκBα (fluorescent red) and cells staining TUNEL-positive (fluorescent green, see arrows) were detected using a 2-color fluorescent microscope (100X magnification) at time 0, 1 h, 2 h, and 6 h after CPT-11 treatment.

REFERENCES

1. Ghosh, S., May, M. J., and Kopp, E. B. NF-kappa B and Rel proteins: evolutionarily conserved mediators of immune responses, Annu Rev Immunol. *16:* 225-60, 1998.

2. Wang, C. Y., Mayo, M. W., Korneluk, R. G., Goeddel, D. V., and Baldwin, A. S., Jr. NF-kappaB antiapoptosis: induction of TRAF1 and TRAF2 and c-IAP1 and c- IAP2 to suppress caspase-8 activation, Science. *281:* 1680-3, 1998.

3. Wang, C. Y., Cusack, J. C., Jr., Liu, R., and Baldwin, A. S., Jr. Control of inducible chemoresistance: enhanced anti-tumor therapy through increased apoptosis by inhibition of NF-kappaB, Nat Med. *5:* 412-7, 1999.

4. Wang, C.-Y., Mayo, M., and Baldwin Jr., A. S. TNF- and cancer therapy-induced apoptosis: potentiation by inhibition of NF-kB, Science. *274:* 784-787, 1996.

5. Bentires-Alj, M., Hellin, A. C., Ameyar, M., Chouaib, S., Merville, M. P., and Bours, V. Stable inhibition of nuclear factor kappaB in cancer cells does not increase sensitivity to cytotoxic drugs, Cancer Res. *59:* 811-5, 1999.

6. Cunningham, D. Current status of colorectal cancer: CPT-11 (irinotecan), a therapeutic innovation, Eur J Cancer. *32A:* S1-8, 1996.

7. Schmitt, C. A. and Lowe, S. W. Apoptosis and therapy, J Pathol. *187:* 127-37, 1999.

8. Wu, G. S. and El-Deiry, W. S. Apoptotic death of tumor cells correlates with chemosensitivity, independent of p53 or bcl-2, Clin Cancer Res. *2:* 623-33, 1996.

9. Kastan, M. B. Molecular determinants of sensitivity to antitumor agents [In Process Citation], Biochim Biophys Acta. *1424:* R37-42, 1999.

10. el-Deiry, W. S. Role of oncogenes in resistance and killing by cancer therapeutic agents, Curr Opin Oncol. *9:* 79-87, 1997.

11. Baldini, N. Multidrug resistance--a multiplex phenomenon [news; comment], Nat Med. *3:* 378-80, 1997.

12. Fisher, D. E. Apoptosis in cancer therapy: crossing the threshold, Cell. *78:* 539-42, 1994.

13. Friesen, C., Herr, I., Krammer, P. H., and Debatin, K. M. Involvement of the CD95 (APO-1/FAS) receptor/ligand system in drug- induced apoptosis in leukemia cells, Nat Med. *2:* 574-7, 1996.

14. Fulda, S., Susin, S. A., Kroemer, G., and Debatin, K. M. Molecular ordering of apoptosis induced by anticancer drugs in neuroblastoma cells, Cancer Res. *58:* 4453-60, 1998.

15. Kirsch, D. G. and Kastan, M. B. Tumor-suppressor p53: implications for tumor development and prognosis, J Clin Oncol. *16:* 3158-68, 1998.

16. Cunningham, D., Pyrhonen, S., James, R. D., Punt, C. J., Hickish, T. F., Heikkila, R., Johannesen, T. B., Starkhammar, H., Topham, C. A., Awad, L., Jacques, C., and Herait, P. Randomised trial of irinotecan plus supportive care versus supportive care alone after fluorouracil failure for patients with metastatic colorectal cancer [In Process Citation], Lancet. *352:* 1413-8, 1998.

17. Kudoh, S., Fujiwara, Y., Takada, Y., Yamamoto, H., Kinoshita, A., Ariyoshi, Y., Furuse, K., and Fukuoka, M. Phase II study of irinotecan combined with cisplatin in patients with previously untreated small-cell lung cancer. West Japan Lung Cancer Group, J Clin Oncol. *16:* 1068-74, 1998.

18. Masuda, N., Fukuoka, M., Takada, M., Kusunoki, Y., Negoro, S., Matsui, K., Kudoh, S., Takifuji, N., Nakagawa, K., and Kishimoto, S. CPT-11 in combination with cisplatin for advanced non-small-cell lung cancer, J Clin Oncol. *10:* 1775-80, 1992.

19. de Forni, M., Bugat, R., Chabot, G. G., Culine, S., Extra, J. M., Gouyette, A., Madelaine, I., Marty, M. E., and Mathieu-Boue, A. Phase I and pharmacokinetic study of the camptothecin derivative irinotecan, administered on a weekly schedule in cancer patients, Cancer Res. *54:* 4347-54, 1994.

20. Oshita, F., Noda, K., Nishiwaki, Y., Fujita, A., Kurita, Y., Nakabayashi, T., Tobise, K., Abe, S., Suzuki, S., Hayashi, I., Kawakami, Y., Matsuda, T., Tsuchiya, S., Takahashi, S., Tamura, T., and Saijo, N. Phase II study of irinotecan and etoposide in patients with metastatic non-small-cell lung cancer, J Clin Oncol. *15:* 304-9, 1997.

21. Cusack, J. C., Spitz, F. R., Nguyen, D., Zhang, W. W., Cristiano, R. J., and Roth, J. A. High levels of gene transduction in human lung tumors following intralesional injection of recombinant adenovirus, Cancer Gene Ther. *3:* 245-9, 1996.

22. Jobin, C., Panja, A., Hellerbrand, C., Iimuro, Y., Didonato, J., Brenner, D. A., and Sartor, R. B. Inhibition of proinflammatory molecule production by adenovirus- mediated expression of a nuclear factor kappaB super-repressor in human intestinal epithelial cells, J Immunol. *160:* 410-8, 1998.

23. Haskill, S., Beg, A. A., Tompkins, S. M., Morris, J. S., Yurochko, A. D., Sampson-Johannes, A., Mondal, K., Ralph, P., and Baldwin, A. S., Jr. Characterization of an immediate-early gene induced in adherent monocytes that encodes I kappa B-like activity, Cell. *65:* 1281-9, 1991.

24. Houghton, P. J., Cheshire, P. J., Hallman, J. C., Bissery, M. C., Mathieu-Boue, A., and Houghton, J. A. Therapeutic efficacy of the topoisomerase I inhibitor 7-ethyl-10-(4-[1-piperidino]-1-piperidino)-carbonyloxy-camptothecin against human tumor xenografts: lack of cross-resistance in vivo in tumors with acquired resistance to the topoisomerase I

inhibitor 9-dimethylaminomethyl-10- hydroxycamptothecin, Cancer Res. *53:* 2823-9, 1993.

25. Kawato, Y., Furuta, T., Aonuma, M., Yasuoka, M., Yokokura, T., and Matsumoto, K. Antitumor activity of a camptothecin derivative, CPT-11, against human tumor xenografts in nude mice, Cancer Chemother Pharmacol. *28:* 192-8, 1991.

26. Cusack, J. C., Wang, C.-Y., Liu, R., and Baldwin, A. S. Chemosensitization of colorectal cancer cells by expression of the NF-kB super-repressor IkBa, Surg Forum. *48:* 815-17, 1997.

27. Liu, Z. G., Hsu, H., Goeddel, D. V., and Karin, M. Dissection of TNF receptor 1 effector functions: JNK activation is not linked to apoptosis while NF-kappaB activation prevents cell death, Cell. *87:* 565-76, 1996.

28. Van Antwerp, D. J., Martin, S. J., Kafri, T., Green, D. R., and Verma, I. M. Suppression of TNF-alpha-induced apoptosis by NF-kappaB [see comments], Science. *274:* 787-9, 1996.

29. Beg, A. A. and Baltimore, D. An essential role for NF-kappaB in preventing TNF-alpha-induced cell death [see comments], Science. *274:* 782-4, 1996.

30. Tartaglia, L. A. and Goeddel, D. V. Two TNF receptors, Immunol Today. *13:* 151-3, 1992.

31. Santana, P., Pena, L. A., Haimovitz-Friedman, A., Martin, S., Green, D., McLoughlin, M., Cordon-Cardo, C., Schuchman, E. H., Fuks, Z., and Kolesnick, R. Acid sphingomyelinase-deficient human lymphoblasts and mice are defective in radiation-induced apoptosis, Cell. *86:* 189-99, 1996.

32. Hsu, H., Huang, J., Shu, H. B., Baichwal, V., and Goeddel, D. V. TNF-dependent recruitment of the protein kinase RIP to the TNF receptor- 1 signaling complex, Immunity. *4:* 387-96, 1996.

33. Hsu, H., Xiong, J., and Goeddel, D. V. The TNF receptor 1-associated protein TRADD signals cell death and NF- kappa B activation, Cell. *81:* 495-504, 1995.

34. Bose, R., Verheij, M., Haimovitz-Friedman, A., Scotto, K., Fuks, Z., and Kolesnick, R. Ceramide synthase mediates daunorubicin-induced apoptosis: an alternative mechanism for generating death signals, Cell. *82:* 405-14, 1995.

35. Kasibhatla, S., Brunner, T., Genestier, L., Echeverri, F., Mahboubi, A., and Green, D. R. DNA damaging agents induce expression of Fas ligand and subsequent apoptosis in T lymphocytes via the activation of NF-kappa B and AP-1, Mol Cell. *1:* 543-51, 1998.

36. Kasibhatla, S., Genestier, L., and Green, D. R. Regulation of fas-ligand expression during activation-induced cell death in T lymphocytes via nuclear factor kappaB, J Biol Chem. *274:* 987-92, 1999.

37. Parker, S. L., Tong, T., Bolden, S., and Wingo, P. A. Cancer statistics, 1997 [published erratum appears in CA Cancer J Clin 1997 Mar-Apr;47(2):68], CA Cancer J Clin. *47:* 5-27, 1997.

38. Eisenberg, B., Decosse, J. J., Harford, F., and Michalek, J. Carcinoma of the colon and rectum: the natural history reviewed in 1704 patients, Cancer. *49:* 1131-4, 1982.

39. Hickman, J. A. Apoptosis induced by anticancer drugs, Cancer Metastasis Rev. *11:* 121-39, 1992.

40. Kerr, J. F., Winterford, C. M., and Harmon, B. V. Apoptosis. Its significance in cancer and cancer therapy [published erratum appears in Cancer 1994 Jun 15;73(12):3108], Cancer. *73:* 2013-26, 1994.

41. Steller, H. Mechanisms and genes of cellular suicide, Science. *267:* 1445-9, 1995.

42. Cunningham, D., Pyrhonen, S., James, R. D., Punt, C. J. A., Hickish, T. S., Heikkila, R., Johannesen, T., Starkhammar, H., Topham, C. A., Ong, E., Herait, P., and Jaques, C. A phase III multicenter randomized study of CPT-11 versus supportive care (SC) alone in patients (Pts) with 5-FU-resistant metastatic colorectal cancer (MCRC)., Proc Am Assoc Clin Oncol. *17:* 1, 1998.

43. Abigerges, D., Chabot, G. G., Armand, J. P., Herait, P., Gouyette, A., and Gandia, D. Phase I and pharmacologic studies of the camptothecin analog irinotecan administered every 3 weeks in cancer patients, J Clin Oncol. *13:* 210-21, 1995.

44. Nieves-Neira, W. and Pommier, Y. Apoptotic response to camptothecin and 7-hydroxystaurosporine (UCN-01) in the 8 human breast cancer cell lines of the NCI Anticancer Drug Screen: multifactorial relationships with topoisomerase I, protein kinase C, Bcl-2, p53, MDM-2 and caspase pathways [In Process Citation], Int J Cancer. *82:* 396-404, 1999.

45. Goldwasser, F., Bae, I., Valenti, M., Torres, K., and Pommier, Y. Topoisomerase I-related parameters and camptothecin activity in the colon carcinoma cell lines from the National Cancer Institute anticancer screen, Cancer Res. *55:* 2116-21, 1995.